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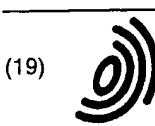
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(19) Europäisches Patentamt
European Patent Office
Office européen des brevets



(11) EP 0 474 727 B1

(12)

EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention
of the grant of the patent:
23.07.1997 Bulletin 1997/30

(21) Application number: **90908809.8**

(22) Date of filing: **18.05.1990**

(51) Int Cl. 6: **C12N 15/12, C12N 1/19,
C12N 1/21, C12N 5/10,
C12N 15/63, C12P 21/02,
C07K 7/06, C07K 7/08,
C07K 14/705, C07K 17/00,
A61K 39/00**

(86) International application number:
PCT/US90/02697

(87) International publication number:
WO 90/14357 (29.11.1990 Gazette 1990/27)

(54) HER2 EXTRACELLULAR DOMAIN

HER2 EXTRAZELLULARE DOMÄNE

DOMAINE EXTRACELLULAIRE DE HER2

(84) Designated Contracting States:
AT BE CH DE DK ES FR GB IT LI LU NL SE

(30) Priority: **19.05.1989 US 354319**

(43) Date of publication of application:
18.03.1992 Bulletin 1992/12

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(56) References cited:
US-A- 4 761 371

- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, October 1987, WASHINGTON US, pages 7159 - 7163; Hudziak, R. et al.: 'Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells.'
- SCIENCE. vol. 232, June 1986, LANCASTER, PA, US, pages 1644 - 1646; AKIYAMA, T. ET AL
- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 82, October 1985, WASHINGTON US, pages 6497 - 6501; Semba, K. et al.: 'A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma.'
- NATURE, vol. 319, 16 January 1986, LONDON GB, pages 226 - 230; Yamamoto, T. et al.: 'Similarity of protein encoded by the human c-erbB-2 gene to epidermal growth factor receptor.'
- NATURE (London, UK), Volume 319, Number 605123, Issued 16 January 1986; YAMAMOTO et al, "Similarity of Protein Encoded by the Human C-Erb-B-2 Gene to Epidermal Growth Factor Receptor", pages 230-234. See Figure 1-3 and final paragraph.

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- **Journal of Cellular Biochemistry, Supplement 13B, Issued January 1989; SHEPARD et al: "p185 Her 2 Monoclonal Antibody has Anti-Proliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor", page 42.**
- **Biochemistry (Washington, US), Volume 26, Number 5, Issued 1987; YARDEN et al: "Epidermal Growth Factor Induces Rapid Reversible Aggregation of the Purified Epidermal Growth Factor Receptor", pages 1443-1451. See materials and methods section.**
- **Biological Abstracts (Philadelphia, US), Volume 87, Number 10, Issued 15 May 1989; HUDZIAK et al: "p185 Her 2 Nonclonal Antibody has Antiproliferative Effects In-Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor", Abstract 105430. See entire document.**
- **Annual Review of Biochemistry (Palo Alto, US), Volume 57, Issued 1988; YARDEN et al: "Growth Factor Receptor Kinases", pages 443-78.**

DescriptionBACKGROUND OF THE INVENTIONField of the Invention

The present invention is generally directed to the extracellular domain of p185^{HER2}, a receptor-like protein which is encoded by the human homolog of the rat *neu* oncogene.

More specifically, the present invention is directed to use of a form of the extracellular domain which is essentially free of transmembrane and cytoplasmic domains for Active Specific Immunotherapy.

Description of Background and Relevant Materials

Human epidermal growth factor receptor 2 (HER2, also known as NGL and human c-erbB-2, or ERBB2), is the human homolog of the rat proto-oncogene *neu*. HER2 encodes a 1,255 amino acid tyrosine kinase receptor-like glycoprotein with homology to the human epidermal growth factor receptor. Although no ligand binding to this probable growth factor receptor has yet been isolated, the HER2 gene product, p185^{HER2}, has the structural and functional properties of subclass I growth factor receptors (Yarden et al., *Ann. Rev. Biochem.*, 57: 443-478 (1988); Yarden et al., *Biochem.*, 27:3113-3119 (1988)).

The receptor tyrosine kinases all have the same general structural motif; an extracellular domain that binds ligand, and an intracellular tyrosine kinase domain that is necessary for signal transduction, or in aberrant cases, for transformation. These 2 domains are connected by a single stretch of approximately 20 mostly hydrophobic amino acids, called the transmembrane spanning sequence. This sequence is thought to play a role in transferring the signal generated by ligand binding from the outside of the cell to the inside. It has also been suggested to play a role in the proper positioning of the receptor in the plasma membrane.

Consistent with this general structure, the p185^{HER2} glycoprotein, which is located on the cell surface, may be divided into three principle portions: an extracellular domain, or ECD (also known as XCD); a transmembrane spanning sequence; and a cytoplasmic, intracellular tyrosine kinase domain. While it is presumed that the extracellular domain is a ligand receptor, as stated above the corresponding ligand has not yet been identified.

The HER2 gene is of particular interest because its amplification has been correlated with certain types of cancer. Amplification of the HER2 gene has been found in human salivary gland and gastric tumor-derived cell lines, gastric and colon adenocarcinomas, and mammary gland adenocarcinomas. Semba et al., *Proc. Natl. Acad. Sci. USA*, 82:6497-6501 (1985); Yokota et al., *Oncogene*, 2:283-287 (1988); Zhou et al., *Cancer Res.*, 47:

6123-6125 (1987); King et al., *Science*, 229:974-976 (1985); Kraus et al., *EMBO J.*, 6:605-610 (1987); van de Vijver et al., *Mol. Cell. Biol.*, 7:2019-2023 (1987); Yamamoto et al., *Nature*, 319:230-234 (1986). Gene

5 transfer experiments have shown that overexpression of HER2 will transform NIH 3T3 cells and also cause an increase in resistance to the toxic macrophage cytokine tumor necrosis factor. Hudziak et al., "Amplified Expression of the HER2/ERBB2 Oncogene Induces Resistance to Tumor Necrosis Factor Alpha in NIH 3T3 Cells", *Proc. Natl. Acad. Sci. USA* 85, 5102-5106 (1988).

Because amplification of the HER2 gene results in greatly increased numbers of the p185^{HER2} protein residing on the surfaces of affected cells, there may be an 15 interrelationship between increased amounts of p185^{HER2} extracellular domain on the surfaces of affected cells and the resistance of these cells to treatment. It would therefore be highly desirable to be able to manipulate the p185^{HER2} extracellular domain in order to 20 investigate several possibilities for the treatment of conditions associated with amplification of the HER2 gene. These therapeutic modes relate not only to the extracellular domain, but also to the putative ligand, which it should be possible to isolate and characterize using the 25 purified p185^{HER2} extracellular domain.

SUMMARY OF THE INVENTION

The present invention is accordingly directed to a 30 composition comprising an extracellular portion of the HER2 molecule containing at least 9 amino acids, and/or containing an immune epitope, which is essentially free of transmembrane and intracellular portions of the HER2 molecule, and in substantially pure form, for use 35 in Active Specific Immunotherapy, and use of said extracellular portion of the HER2 molecule in the manufacture of a composition for treatment of a patient by Active Specific Immunotherapy. The extracellular portion may be at least about 99% pure, and may extend 40 to the entire extracellular portion of the HER2 molecule. Moreover, the extracellular portion may be antigenic in animals, and may be conjugated with a peptide having immunogenic properties; this peptide may contain an 45 immune epitope.

The extracellular portion of the HER2 molecule may be combined with suitable adjuvants.

BRIEF DESCRIPTION OF FIGURES

50 Fig 1. HER2 expression vector and full-length and mutant HER2 proteins. The HER2 expression vector contained eukaryotic transcriptional units for the mouse dihydrofolate reductase (DHFR) cDNA and the bacterial neomycin phosphotransferase (neo) gene, both under SV40 early promoter control. Transcription of the full-length HER2 cDNA was also driven by the early SV40 promoter. The full-length HER2 protein contains an extracellular domain with two cysteine-rich clusters

(hatched rectangle), separated by the transmembrane-spanning region (filled rectangle) from the intracellular tyrosine kinase domain (open rectangle). The mutant protein p185^{HER2ΔTM} has a deletion of 28 amino acids, including the transmembrane-spanning region. The truncated p185^{HER2XCD} protein contains all N-terminal sequences up to 8 amino acids before the transmembrane-spanning region.

Fig. 2. Amplification of HER2 and HER2^{ΔTM} genes. Cell lines transfected with plasmids expressing wild type or the ΔTM mutant HER2 cDNAs were amplified to resistance to 400 nM methotrexate. Cultures were metabolically labeled with [³⁵S]-methionine and proteins immunoprecipitated with the G-H2CT17 antibody. Lane 1: CVN-transfected NIH 3T3 vector control line. Lanes 2 and 3: Parental and amplified HER2-3 line. Lanes 4, 5, and 6, 7: Parent and amplified lines derived from two independent clones, A1 and B2, of the ΔTM mutant. The arrows indicate the positions expected for proteins of apparent molecular mass of 175 and 185 kDa.

Fig. 3. Autophosphorylation of p185^{HER2} and p185^{HER2ΔTM} proteins. Triton X-100 lysates of control, HER2-3₄₀₀, and ΔTM-expressing cell lines were prepared and immunoprecipitated with the G-H2CT17 antibody. The immune complexes were incubated in 50 μl of HNTG, 5 mM MnCl₂ with 3 uCi [γ -³²P] for 20 min, electrophoresed on a 7.5% polyacrylamide gel, and labeled bands visualized by autoradiography. Lane 1: CVN vector control. Lane 2: HER2-3₄₀₀ cells expressing full-length HER2. Lanes 3 and 4: Two independent lines expressing p185^{HER2ΔTM}. The arrows indicate the positions expected for proteins of apparent molecular mass of 66.2, 97, 175, and 185 kDa.

Fig 4. Secretion assay of ΔTM mutants. Cell lines CVN, HER2-3₄₀₀, ΔTM-A1₄₀₀, and ΔTM-B2₄₀₀ were labeled with [³⁵S]-methionine overnight. Triton X-100 cell extracts were prepared and the labeling medium collected. Cells and cell-conditioned media were immunoprecipitated with G-H2CT17 antibody and analyzed on 7.5% SDS-PAGE gels. Lanes 1-4 are immunoprecipitations of cell extracts from the various lines, and lanes 5-8 are immunoprecipitations from the corresponding cell-conditioned media. Lanes 1 and 5: CVN vector control. Lanes 2 and 6: HER2-3₄₀₀ cell lines expressing full-length p185^{HER2}. Lanes 3, 4 and 7, 8: ΔTM-A1₄₀₀ and ΔTM-B2₄₀₀ cell lines expressing mutant p185^{HER2ΔTM}. The arrows indicate the positions expected for proteins of apparent molecular mass of 175 and 185 kDa.

Fig 5. Secretion of p185^{HER2XCD} from 3T3 and CHO cells. NIH 3T3 and CHO cell lines expressing full-length and truncated p185^{HER2} and vector controls were labeled with [³⁵S]-methionine overnight. Cell extracts and cell-conditioned media were immunoprecipitated with anti-HER2 monoclonal antibody 3E8 and analyzed on 7.5% SDS-PAGE gels. Lanes 1 and 2: NIH 3T3 control cell line, extract and conditioned medium. Lanes 3 and 4: NIH 3T3 line A1 expressing p185^{HER2XCD}, cells and medium. Lanes 5 and 6: NIH 3T3 line 3₄₀₀ expressing

full-length p185^{HER2}, cells and conditioned medium. Lanes 7 and 8: CHO control line, cell extract and conditioned medium. Lanes 9 and 10: CHO line 2, expressing p185^{HER2XCD} cells and conditioned medium. Lanes 11 and 12: CHO line HER2₅₀₀, expressing full-length p185^{HER2}, cells and conditioned medium. The arrows indicate the molecular mass of the indicated protein bands.

Fig 6. Increase in expression of p185^{HER2XCD} with amplification. The CHO-derived cell line HER2XCD-2 was selected for growth in 500 nM and then 3000 nM methotrexate. The parent line, the two amplified derivatives, and control vector-transfected cells were labeled with [³⁵S]-methionine. Cell extracts and cell-conditioned media were immunoprecipitated with the anti-HER2 monoclonal antibody 3E8 and analyzed on a 7.5% SDS-PAGE gel. Lanes 1 and 2: CVN cell extract and conditioned medium. Lanes 3 and 4: HER2XCD-2, unamplified cells and conditioned medium. Lanes 5 and 6: HER2XCD-2 amplified to resistance to 500 nM methotrexate, cells and conditioned medium. Lanes 7 and 8: HER2XCD-2 amplified to resistance to 3000 nM methotrexate, cells and conditioned medium. For comparative purposes, one-fifth as much sample of the 3000 nm line was loaded compared to the control, 0 nM, and 500 nM lines. The band intensities were quantitated with an LKB2202 laser densitometer. The arrows show the positions of proteins of apparent molecular mass of 88 and 103 kDa.

Fig 7. Biosynthesis of p185^{HER2XCD}. The CHO line HER2XCD2₃₀₀₀ was labeled with [³⁵S]-methionine and cell extracts, and cell-conditioned media prepared. Lanes 1 and 2: Cell extract and cell-conditioned medium. Lanes 3 and 4: The same conditioned medium incubated or mock-incubated with endo H. Lanes 5 and 6: Cell extract and conditioned medium from cells treated with tunicamycin. The arrows show the positions expected for proteins of apparent molecular mass of 73, 88, and 103 kDa.

Fig 8. Morphology of NIH 3T3 cells transfected with HER2 and HER2^{ΔTM} expression constructs. A and D: Parental and amplified cells from NIH 3T3 cells transfected with vector alone. B and E: NIH 3T3 cells expressing p185^{HER2ΔTM} (line A1), parent and amplified derivative selected for resistance to 400 nM methotrexate. C and F: NIH 3T3 cells expressing wild type p185^{HER2} (line 3), parent and amplified derivative selected for resistance to 400 nM methotrexate.

Fig 9. Cell surface and cytoplasmic immunofluorescence staining of control, HER2, and HER2^{ΔTM} lines. The top photos are intact cells labeled with anti-HER2 monoclonal antibody. The bottom photos are the same cell lines treated with 0.15% Triton X-100 detergent before addition of antibody. A and D: Control NIH 3T3 cells transfected with vector only. B and E: Cell line HER2 ΔTM-A1₄₀₀, expressing p185^{HER2ΔTM}. C and F: Cell line HER2-3₄₀₀ expressing p185^{HER2}.

Fig 10. Fluorescence-activated cell sorter histo-

grams of control, HER2 and HER2 Δ TM cells binding anti-p185^{HER2} monoclonal antibody 4D5. Binding by the control antibody, 368, directed against human tissue plasminogen activator, light, broken line. Binding by the anti-HER2 antibody 4D5, dark unbroken line. Panel A: Control NIH 3T3 cells transfected with vector only. Panel B: Cell line HER2-3₄₀₀, expressing p185^{HER2}. Panel C: Cell line HER2 Δ TMA1₄₀₀ expressing p185 ^{Δ TM}.

Fig 11. Biosynthesis of p185^{HER2} and p185^{HER2 Δ TM} proteins. Cell lines HER2-3₄₀₀ and HER2 Δ TMA1₄₀₀ were labeled with [³⁵S]-methionine and p185^{HER2} and p185^{HER2 Δ TM} proteins collected by immunoprecipitation and analyzed on a 7.5% SDS-PAGE gel. Lane 1: Vector control. Lane 2: Untreated p185^{HER2 Δ TM}. Lanes 3 and 4: Aliquots of the same cell extract treated or mock-treated with endo H. Lane 5: Nonglycosylated p185^{HER2} from cells treated with tunicamycin. Lane 6: Untreated p185^{HER2}. Lanes 7 and 8: Aliquots of the same cell extract treated or mock-treated with endo H. Lane 9: Non-glycosylated p185^{HER2 Δ TM} from cells treated with tunicamycin. The arrows show the positions of proteins of apparent molecular weight of 175 and 185 kDa.

Fig. 12. Purification of the HER2 extracellular domain. Purified HER2 extracellular domain samples were analyzed using PhastSystem SDS-Gel electrophoresis and silver stained protocols as recommended by Pharmacia. SDS polyacrylamide gel (10-15% gradient) electrophoretic analysis was performed according to Pharmacia protocol File No. 110. Silver staining was performed according to Pharmacia protocol File No. 210. Lane 1 contains molecular weight markers (BRL). Lane 2: Chinese Hamster Ovary Cell 15 X concentrate (1 microliter). Lanes 3 and 4: immunoaffinity purified HER2 extracellular domain (1.6 micrograms and 0.16 microgram, respectively). Lanes 5 and 6: immunoaffinity purified HER2 extracellular domain after DEAE chromatography (0.25 micrograms and 0.083 micrograms, respectively). Lanes 7 and 8: HER2 extracellular domain after formulation in PBS (0.32 micrograms and 0.082 micrograms, respectively).

Fig. 13. The predicted amino acid sequence of the HER2 extracellular domain, with the corresponding nucleic acid sequence. The boxed sequences show potential T-cell epitopes, using the algorithm developed by Margolit *et al.*, J. Immunol. 138:2213-2229(4) (1987).

DETAILED DESCRIPTION

It was initially hypothesized that removal of the transmembrane spanning sequence would yield a protein which would be secreted from the cell. As previously indicated, the transmembrane spanning sequence is principally composed of hydrophobic amino acids, which effectively anchor the protein in the cell membrane. Removal of this sequence would therefore be expected to permit passage of the protein through the membrane.

A first construct was accordingly prepared which

deleted exactly in-frame the 22 amino acid transmembrane spanning sequence of HER2, and 3 amino acids on either side (Figure 1). The construct was prepared as follows:

5 The central EcoRI fragment containing the transmembrane spanning segment was cloned into the EcoRI site of the bacteriophage vector M13 mp18 (Yanisch-Perron *et al.*, Gene, 33:103-119 (1985). The non-coding strand was used as template for oligonucleotide-directed mutagenesis. The construct deleted the transmembrane spanning sequence, and an additional 3 amino acids before and after.

10 Residues 651-678 were deleted by priming double stranded DNA synthesis with a 30 base pair oligonucleotide of sequence 5' CAG AGA GCC AGC CCT CAG CAG AAG ATC CGG 3'. The double stranded DNA was transformed into SR101 cells and mutants identified by hybridization to the same oligonucleotide 5' end labeled by polynucleotide kinase and [γ -³²P] ATP (Amersham, 20 5000 Ci/mmol). An EcoRI fragment containing the deletion was recombined into a plasmid expressing the HER2 cDNA, replacing the wild type sequence.

15 When expressed in NIH 3T3 cells, this mutant, designated HER2 Δ TM, produced a polypeptide, designated p185^{HER2 Δ TM}, of apparent molecular weight 175 kD (Figure 2, lanes 5 and 7). Production took place at levels comparable to wild type p185^{HER2} amplified to the same level of resistance to methotrexate (Figure 2, lane 3). The mutant proteins also retained an active tyrosine kinase activity.

20 In the presence of [γ -³²P]-ATP, the mutant proteins (Figure 3, lanes 3 and 4) were autophosphorylated to the same extent as unaltered p185^{HER} (Figure 3, lane 2). Figure 3 also shows autophosphorylated p185^{HER2 Δ TM}-related proteins of lower molecular weight than the complete protein. These smaller proteins may represent degradation products and, since they are not observed with p185^{HER2}, could imply a difference in intracellular processing of the mutant form.

25 To determine whether the form lacking the transmembrane sequence was secreted, cells were metabolically labeled with ³⁵S-methionine. The culture conditions used herein were as follows: cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 supplemented with glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 ug/ml), and 10% serum. NIH 3T3-derived cell lines were cultured with calf serum (Hyclone). Chinese Hamster Ovary cells deficient in dihydrofolate reductase (CHO-DHFR) were cultured in fetal bovine serum (Gibco) supplemented with glycine (0.13 mM), hypoxanthine (0.11 mM), and thymidine (0.02 mM). (For selection of the transfected plasmid DHFR gene or to amplify introduced plasmids by methotrexate selection, the glycine, hypoxanthine, and thymidine were omitted and extensively dialyzed serum substituted for fetal bovine serum.)

30 Both cells and cell-conditioned medium were as-

sayed for p185^{HER2}. Figure 4 demonstrates that all p185^{HER2} remained cell associated (lanes 2, 3, 4), and neither the wild type protein nor the mutant form was secreted (lanes 6, 7, 8).

Thus, contrary to expectations, deletion of the transmembrane spanning sequence was not sufficient to yield a secreted form of p185^{HER2}.

The discovery that p185^{HER2ΔTM} is not secreted suggested that perhaps there are sequences distal to the transmembrane spanning region that prevent passage of p185^{HER2} through the plasma membrane. A second mutant was accordingly made that contained a UAA stop codon 8 amino acids before the beginning of the proposed transmembrane spanning sequence (Figure 1).

The second construct truncated p185^{HER2} 8 amino acids before the start of the transmembrane spanning region at residue 645 by addition of a polypeptide chain-terminating TAA codon. The oligonucleotide 5' AAG GGC TGC CCC GCC GAG TAA TGA TCA CAG AGA GCC AGC CCT 3' was used to prime synthesis of double-stranded DNA from the same template used to construct the ΔTM mutant. Mutant plaques were identified by hybridization to the 5' end-labeled oligonucleotide, and confirmed by checking for the presence of a Bcl 1 site also introduced directly after the ochre codon. The chain-terminated mutant, designated HER2^{XCD}, was then recombined into the HER2 cDNA expression plasmid. The structure of the plasmid and the 2 mutant HER2 derivatives is shown in Figure 1.

Secretion of the resulting form of p185^{HER2}, designated p185^{HER2XCD}, was assayed by first metabolically labeling the cells with ³⁵S-methionine, followed by immunoprecipitation of p185^{HER2}-related proteins from both the cells and cell-conditioned media. In the immunoprecipitation procedure (Hudziak et al., *Proc. Natl. Acad. Sci. USA*, 84:7159-7163 (1987)), cells were harvested by trypsinization, counted electronically with a Coulter counter, and plated at least 6 hrs. before labeling. The plating medium was removed, cells washed with PBS, and the cells re-fed with methionine-free Dulbecco's modified minimal medium. [³⁵S]-methionine (Amersham, 800 Ci/mmol, 29.6 TBq/mmol) was added at 100 uCi/6 cm plate in a volume of 3 ml. Cells were lysed at 4°C with 0.4 ml of HNEG lysis buffer per 6 cm plate. After 10 min, 0.8 ml of lysis dilution buffer (HNEG buffer with 1% bovine serum albumin, 0.1% Triton X-100 detergent) was added to each plate and the extracts were clarified by microcentrifugation for 5 min. Medium to be assayed for secretion of p185^{HER2} related proteins was collected and clarified by microcentrifugation.

Antibodies were added to cell extracts or conditioned medium and allowed to bind at 4°C for 2-4 h. The polyclonal antibody, G-H2CT17(0), recognizing the carboxy-terminal 17 amino acids of p185^{HER2}, was used for characterization of cell lines expressing the transmembrane-deleted form of p185^{HER2}. The monoclonal antibody 3E8, recognizing an epitope on the extracellu-

lar domain (Hudziak et al., *Mol. Cell. Bio.*, 9:1165-1172 (1989)), was used at 8 ug/reaction to immunoprecipitate the truncated form. Seven ug of rabbit anti-mouse IgG was added to immunoprecipitations using this monoclonal to improve its binding to protein A-sepharose. Immune complexes were collected by absorption to protein A-sepharose beads and washed (Hudziak et al., *Proc. Natl. Acad. Sci. USA*, 85:5102-5106 (1988); Hudziak et al., *Proc. Natl. Acad. Sci. USA*, 84:7159-7163 (1987)). Proteins were separated on 7.5% sodium dodecyl sulphate-polyacrylamide gels (SDS-PAGE) and analyzed by autoradiography.

This revealed a form of p185^{HER2XCD} of M_r 88,000 kD that is associated with the cells (Figure 5, lanes 3 and 9); however, the cell-conditioned media from both the NIH 3T3 cells and Chinese hamster ovary-derived lines also contains larger amounts of a protein of M_r 103,000, which is immunoprecipitated by anti-HER2 monoclonal antibody (Figure 5, lanes 4 and 10). Full length p185^{HER2} was also expressed in both NIH 3T3 and CHO cells (Figure 5), lanes 5 and 11. There is no secretion of native p185^{HER2} from either of these cell types (Figure 5, lanes 6 and 12).

The larger size of the observed proteins in the cells and cell-conditioned medium (88,000 and 103,000, respectively) compared to the size predicted by the amino acid sequence (71,644) suggested that the truncated form was being glycosylated.

This was confirmed by treating the cells with the antibiotic tunicamycin, which prevents N-linked glycosylation. Treatment with tunicamycin resulted in the appearance of a cell-associated protein of M_r 73,000, which is close to that predicted by the amino acid sequence (Figure 7, lane 5). It also almost completely inhibited secretion of p185^{HER2XCD} into the medium (Figure 7, lane 6). Cell-conditioned medium from tunicamycin treated cells contains only small amounts of the mature 103,000 form, and none of the smaller forms (lane 6). This further suggests that secretion of p185^{HER2XCD} is coupled to glycosylation.

The extent of glycosylation of the secreted form was investigated with the enzyme endoglycanase H (endo H, Boehringer Manheim). This enzyme will hydrolyze asparagine-linked oligosaccharides of the high mannose type. High mannose oligosaccharides are biosynthetic intermediates in the glycosylation process. Final maturation of the carbohydrate side chains involves trimming off some mannose and addition of other sugars such as fucose. Such mature oligosaccharide side chains are resistant to endo H.

To determine if secreted p185^{HER2XCD} is resistant to this enzyme, cell conditioned medium labeled with ³⁵S-methionine was immunoprecipitated. The immunoprecipitates were collected onto protein A sepharose beads and incubated with endo H. Neither mock incubated (lane 3) nor endo H-treated p185^{HER2XCD} (lane 4) showed any decrease in mobility associated with hydrolysis of the glycosyl side chains, demonstrat-

ing that the glycosylation is complete.

Without being bound by any particular theory, these results taken together suggest that the cell-associated form of p185^{HER2XCD} is an intermediate, and that fully mature glycosylated p185^{HER2} extracellular domain is being synthesized and secreted. The lack of secretion of the p185^{HER2ΔTM} protein could be hypothesized to result from the presence of processing information in the transmembrane spanning sequence which is necessary for Golgi transport and targeting of the plasma membrane; however, from these studies it appears instead that transport of tyrosine kinase receptor (or receptor-like) extracellular domain to the cell surface is coupled to proper glycosylation.

Therefore, insertion of the UAA stop codon 8 amino acids before the beginning of the proposed transmembrane spanning sequence yields a fully mature glycosylated p185^{HER2} extracellular domain which is freely secreted by the cell.

Having succeeded in producing a secreted form of p185^{HER2}, the next stage involved investigating whether the amount of secreted protein could be increased by gene amplification. Using the CHO-derived cell line, it was found that the amount of extracellular domain could be increased by methotrexate selection. The amount of secreted product increased 29-fold in cells selected for resistance to 500 nm methotrexate, and a further 4.4-fold by selection for resistance to 3000 nm methotrexate (Fig. 6).

Thus, a total increase of 128-fold in secreted p185^{HER2XCD} was obtained when this cell line was amplified to resistance to 3000 nm methotrexate, making the production of relatively large quantities of p185^{HER2XCD} possible.

To determine whether overexpression of p185^{HER2ΔTM} results in cell transformation, DNA was introduced in mammalian cells by the CaHPO₄ coprecipitation method (Graham *et al.*, *Virology*, 52:456-467 (1973)). Five ug of plasmid DNA was added to half-confluent plates of cells (6.0 cm) in 1 ml for 4-6 h. The DNA was removed and the cells shocked with 20% (vol/vol) glycerol. After 2 days for phenotypic expression the selective agent geneticin was added at 400 ug/ml. Clones were picked using glass cloning cylinders with petroleum jelly for the bottom seal. The introduced plasmids were amplified by the methotrexate selection procedure (Kaufman *et al.*, *J. Mol. Biol.*, 159:601-621 (1982)).

When the ΔTM mutant was expressed in NIH 3T3 cells, primary unamplified colonies after selection had the normal flat nontransformed phenotype (Figure 8, compare photo B with vector control alone, photo A). After the expression level was increased by methotrexate selection, the cells took on the refractile, spindle-shaped appearance of transformed cells and also grew piled up in irregular clumps (photo E). This observation is similar to our earlier findings with the unaltered HER2 cDNA (photos C and F, parent and amplified derivatives respectively), and suggests that high levels of expres-

sion of the mutant ΔTM protein were also transforming.

The morphological changes seen at equivalent levels of amplification (400 nm methotrexate) are not as marked for the mutant, implying that the transforming potential of this form of p185^{HER2} may be less. At higher levels of resistance to methotrexate, the ΔTM cells become even more transformed in appearance.

The plasmid was also negative in a focus-forming assay whereas the wild type HER2 plasmid was positive, further indicating that the transforming potential of p185^{HER2ΔTM} protein is lower. Cells expressing high levels also displayed another property of the transformed phenotype, growth in soft agar. Colony formation in soft agar was determined by harvesting each line to be assayed with trypsin, counting the cells (Coulter counter), and plating 80,000 cells per 6-cm dish. The top layer consisted of 4 ml of 0.25% agar (Difco, "purified") over a bottom layer of 5 ml of 0.5% agar. Colonies were counted after 3-4 weeks. Cells from 2 independent clones plated in soft agar gave rise to soft agar colonies with an efficiency comparable to cells expressing the wild type HER2 gene:

Table I

Soft Agar Colony Formation	
Cell Line	# of Soft Agar Colonies
CVN	0
CVN ₄₀₀	0
HER2-3 ₀	5 +/- 1
HER2-3 ₄₀₀	208 +/- 27
ΔTM-A1 ₀	0
ΔTM-A1 ₄₀₀	205 +/- 62
ΔTM-B2 ₀	0
ΔTM-B2 ₄₀₀	205 +/- 13

Two control lines were used; NIH 3T3 cells transfected with a plasmid expressing only the neo and DHFR genes, and the same line amplified to resistance to 400 nM methotrexate. The number of soft agar colonies arising was determined for both parental and amplified lines of clones expressing either p185^{HER2} or p185^{HER2ΔTM} proteins. Each cell line was plated in triplicate and the value averaged.

Therefore, according to the present invention it has been determined that removal of only the transmembrane spanning sequence does not lead to secretion of p185^{HER2}, unless the entire tyrosine kinase domain is also deleted. Removal of this domain results in proper glycosylation and secretion of the extracellular domain.

In order to obtain purified HER2 extracellular domain working material, Chinese Hamster Ovary Cell Harvest Fluid (CFF) containing recombinant HER2 ECD may be first concentrated by ultrafiltration, and then purified by immunoaffinity chromatography using a HER2 specific MAb coupled to CNBr activated Sepharose; other suitable immobilization supports may be used.

Concentrated CCF is applied to the affinity column after filtration through a 0.2 micron Millipore filter. Purification cycles are performed as necessary until the desired amount of CCF is processed.

During each cycle of purification, the concentrated CCF is applied and the affinity column is washed to baseline with 0.5 M Tris buffer containing 0.15 M NaCl at a pH of approximately 7.5 (TB). HER2 extracellular domain is then eluted from the column with 0.1 M sodium citrate buffer containing 0.5 M NaCl at a pH of approximately 3.5. The affinity column eluant fractions containing HER2 ECD are pooled and neutralized. The immunoaffinity column is reequilibrated between each purification cycle with TB.

In a second step, the affinity column eluant is buffer exchanged into 25 ml of Tris buffer, at a pH of approximately 7.0 (TB2). The HER2 extracellular domain is then applied to a DEAE Sepharose Fast Flow column, and washed with TB2. The HER2 ECD is removed from the column by step or gradient salt elution in TB2 (containing up to 200 mM NaCl).

After DEAE chromatography, purified HER2 ECD fractions are pooled, exchanged into phosphate-buffered saline, and stored at 2-8° C. The resulting material is substantially pure, i.e., about 99% pure (see Fig. 12).

By means of the present invention it is accordingly possible to produce a secreted, glycosylated p185^{HER2} extracellular domain. This opens several possibilities for further research, as well as a broad range of potential therapeutic applications.

As previously stated, the HER2 gene is of particular interest because its amplification has been correlated with certain types of cancer. In a survey of 189 primary mammary gland adenocarcinomas, it was found that 30% contained amplifications of the HER2 gene. Slamon et al., "Human Breast Cancer: Correlation of Relapse and Survival with Amplification of the HER-2/neu Oncogene," *Science* 235, 177-182 (1987). Amplification was correlated with a negative prognosis and high probability of relapse.

This suggests that of the 120,000 women diagnosed with breast cancer each year, 36,000 will have HER2 amplification. Approximately half of these women, or about 15,000, may be expected to exhibit greater than 5-fold amplification, corresponding to nearly half of the 40,000 breast cancer-related deaths each year.

It has been demonstrated that a monoclonal antibody directed against the p185^{HER2} extracellular domain specifically inhibits growth of breast tumor-derived cell lines overexpressing the HER2 gene product; prevents HER2-transformed NIH 3T3 cells from forming colonies in soft agar; and reduces the resistance to the cytotoxic effect of tumor necrosis factor alpha which accompanies HER2 overexpression. Hudziak et al., "p185^{HER2} Monoclonal Antibody has Antiproliferative Effects In Vitro and Sensitizes Human Breast Tumor Cells to Tumor Necrosis Factor", *Mol. Cell. Biol.* 9: 1165-1172 (1989). See also, Drebin et al., "Inhibition of

Tumor Growth by a Monoclonal Antibody Reactive with an Oncogene-Encoded Tumor Antigen", *Proc. Natl. Acad. Sci. USA* 83, 9129-9133 (1986) (*in vivo* treatment with anti-p185 monoclonal antibody asserted to inhibit

5 tumorigenic growth of neu-transformed NIH 3T3 cells implanted in mice).

This effect presents the possibility that conditions characterized by amplification of the HER2 gene may be subject to treatment via Active Specific Immunotherapy. This therapeutic modality contemplates provoking an immune response in a patient by vaccination with an immunogenic form of the extracellular domain. The extracellular domain (or a derivative thereof, as discussed below) may be combined with a local adjuvant

15 which is safe and effective in humans, such as alum, Bacillus calmette-Guerin (BCG), adjuvants derived from BCG cell walls, Detox (Ribi-immunochem), Syntex-1, or Corynebacterium parvum. Alternatively, systemic adjuvants, such as Interferon gamma, Interleukin 1, Interleukin 2, or Interleukin 6 may be suitable. An appropriate dose and schedule would be selected to maximize humoral and cell-mediated response.

It may also be possible to enhance an immune response by targeting the immunogen to the immune system, 25 which could lead to more efficient capture of the antigen by antigen presenting cells, or by directing the immunogen so that it is presented by MHC Class 1 molecules, since these usually induce a T-cell response.

In addition to Active Specific Immunotherapy, it 30 should be possible to use the purified extracellular domain to isolate and characterize the putative ligand. The HER2 ligand may be used in turn to deliver toxin to tumor cells which are overexpressing HER2, such as by molecular fusion of the ligand with toxin, or by chemical cross-linking. Alternatively, patients overexpressing 35 HER2 may be vaccinated with HER2 ligand conjugated to, or in combination with, a suitable adjuvant.

A patient overexpressing HER2 will also presumably be overexpressing the HER2 ligand. The ligand- 40 HER2 binding interaction, which is likely to contribute to tumor growth, may be inhibited by blocking free ligand in the patient's serum. This blocking can be accomplished by treating the patient with the HER2 extracellular domain, which will proceed to bind free HER2 ligand, thereby preventing the ligand from binding to the 45 HER2 receptor site.

Rather than using the HER2 extracellular domain *per se*, it may be more desirable to use a derivative which has an increased affinity for the ligand, and/or 50 which has an increased half-life *in vivo*. Cross-linking on cells is known to improve binding affinity, suggesting that artificial cross-linking can be used to improve the binding ability of the HER2 extracellular domain. The half-life of the extracellular domain in serum can be improved by, for example, fusing the extracellular domain with other molecules present in the serum which are known to have a long half-life, such as the Fc-portion of 55 an immunoglobulin molecule.

The present invention has of necessity been discussed herein by reference to certain specific methods and materials. It is to be understood that the discussion of these specific methods and materials in no way constitutes any limitation on the scope of the present invention, which extends to any and all alternative materials and methods suitable for accomplishing the ends of the present invention.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE

1. A composition comprising an extracellular portion of the HER2 molecule comprising at least 9 amino acids and/or an immune epitope, essentially free of transmembrane and intracellular portions of said HER2 molecule, and in substantially pure form, for use in Active Specific Immunotherapy.
2. A composition according to claim 1, wherein the extracellular portion of the HER2 molecule has a purity of at least about 99%.
3. A composition according to claim 1 or claim 2, comprising the entire extracellular portion of the HER2 molecule.
4. A composition according to any one of the preceding claims, wherein the extracellular portion of the HER2 molecule is conjugated with a peptide having immunogenic properties.
5. A composition according to claim 4, wherein said peptide comprises an immune epitope.
6. A composition according to any one of claims 1 to 5 further comprising an adjuvant.
7. A composition according to claim 6 wherein the adjuvant comprises any of alum, Bacillus calmette-Guerin (BCG), a BCG cell wall derivative, Detox, Corynebacterium parvum, interferon gamma, interleukin 1, interleukin 2, Syntex-1 and interleukin 6.
8. Use of an extracellular portion of the HER2 molecule comprising at least 9 amino acids and/or an immune epitope, essentially free of transmembrane and intracellular portions of said HER2 molecule, in the manufacture of a composition for treatment of a patient by Active Specific Immunotherapy.
9. Use according to claim 8, wherein the composition comprises the entire extracellular portion of the HER2 molecule.

5 10. Use according to claim 8 or claim 9 wherein the extracellular portion of the HER2 molecule is conjugated with a peptide having immunogenic properties.

11. Use according to claim 10 wherein said peptide comprises an immune epitope.

12. Use according to any one of claims 8 to 11, wherein the composition comprises an adjuvant.

15 13. Use according to claim 12, wherein the adjuvant comprises any of alum, Bacillus calmette-Guerin (BCG), a BCG cell wall derivative, Detox, Corynebacterium parvum, interferon gamma, interleukin 1, interleukin 2, Syntex-1 and interleukin 6.

Claims for the following Contracting State : ES

- 20 1. A method for the manufacture of a composition for use in Active Specific immunotherapy, comprising the manufacture of an extracellular portion of the HER2 molecule comprising at least 9 amino acids and/or an immune epitope, essentially free of transmembrane and intracellular portions of said HER2 molecule, and in substantially pure form.
- 25 2. A method according to claim 1, wherein the extracellular portion of the HER2 molecule has a purity of at least about 99%.
- 30 3. A method according to claim 1 or claim 2, wherein the extracellular portion of the HER2 molecule comprises the entire extracellular portion of the HER2 molecule.
- 35 4. A method according to any one of the preceding claims wherein the extracellular portion of the HER2 molecule is conjugated with a peptide having immunogenic properties.
- 40 5. A method according to claim 4, wherein said peptide comprises an immune epitope.
- 45 6. A method according to any one of claims 1 to 5 wherein the composition further comprises an adjuvant.
- 50 7. A method according to claim 6 wherein the adjuvant comprises any of alum, Bacillus calmette-Guerin (BCG), a BCG cell wall derivative, Detox, Corynebacterium parvum, interferon gamma, interleukin 1, interleukin 2, Syntex-1 and interleukin 6.
- 55 8. The use of an extracellular portion of the HER2 molecule comprising at least 9 amino acids and/or an immune epitope, essentially free of transmembrane

- and intracellular portions of said HER2 molecule, in the manufacture of a composition for treatment of a patient by Active Specific Immunotherapy.
9. The use according to claim 8 wherein the extracellular portion of the HER2 molecule comprises the entire extracellular portion of the HER2 molecule.
10. The use of claim 8 or claim 9 wherein the extracellular portion of the HER2 molecule is conjugated with a peptide having immunogenic properties.
11. The use according to claim 10, wherein said peptide comprises an immune epitope.
12. The use according to any one of claims 8 to 11, wherein the composition comprises an adjuvant.
13. The use according to claim 12, wherein the adjuvant comprises any of alum, Bacillus calmette-Guerin (BCG), a BCG cell wall derivative, Detox, Corynebacterium parvum, interferon gamma, interleukin 1, interleukin 2, Syntex-1 and interleukin 6.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE

1. Zusammensetzung, umfassend einen extrazellulären Abschnitt des HER2-Moleküls mit zumindest 9 Aminosäuren und/oder einem Immunepitop, der im wesentlichen frei von Transmembran- und intrazellulären Abschnitten des HER2-Moleküls und in im wesentlichen reiner Form ist, zur Verwendung bei der aktiven spezifischen Immuntherapie.
2. Zusammensetzung nach Anspruch 1, worin der extrazelluläre Abschnitt des HER2-Moleküls eine Reinheit von zumindest etwa 99% besitzt.
3. Zusammensetzung nach Anspruch 1 oder 2, umfassend den gesamten extrazellulären Abschnitt des HER2-Moleküls.
4. Zusammensetzung nach einem der vorhergehenden Ansprüche, worin der extrazelluläre Abschnitt des HER2-Moleküls mit einem Peptid, das immunogene Eigenschaften aufweist, konjugiert ist.
5. Zusammensetzung nach Anspruch 4, worin das Peptid ein Immunepitop umfasst.
6. Zusammensetzung nach einem der Ansprüche 1 bis 5, die weiters ein Adjuvant umfaßt.

7. Zusammensetzung nach Anspruch 6, worin das Adjuvant ein beliebiges aus Alaun, Bacillus Calmette-Guerin (BCG), ein BCG-Zellwandderivat, Detox, Corynebacterium parvum, Interferon-γ, Interleukin 1, Interleukin 2, Syntex-1 und Interleukin 6 umfasst.
8. Verwendung eines extrazellulären Abschnitts des HER2-Moleküls mit zumindest 9 Aminosäuren und/oder einem Immunepitop, der im wesentlichen frei von Transmembran- und intrazellulären Abschnitten des HER2-Moleküls ist, bei der Herstellung einer Zusammensetzung zur Behandlung eines Patienten durch aktive spezifische Immuntherapie.
- 15 9. Verwendung nach Anspruch 8, worin die Zusammensetzung den gesamten extrazellulären Abschnitt des HER2-Moleküls umfasst.
- 10 10. Verwendung nach Anspruch 8 oder 9, worin der extrazelluläre Abschnitt des HER2-Moleküls mit einem Peptid, das immunogene Eigenschaften aufweist, konjugiert ist.
11. Verwendung nach Anspruch 10, worin das Peptid ein Immunepitop umfasst.

12. Verwendung nach einem der Ansprüche 8 bis 11, worin die Zusammensetzung ein Adjuvant umfasst.
- 30 13. Verwendung nach Anspruch 12, worin das Adjuvant ein beliebiges aus Alaun, Bacillus Calmette-Guerin (BCG), ein BCG-Zellwandderivat, Detox, Corynebacterium parvum, Interferon-γ, Interleukin 1, Interleukin 2, Syntex-1 und Interleukin 6 umfasst.

Patentansprüche für folgenden Vertragsstaat : ES

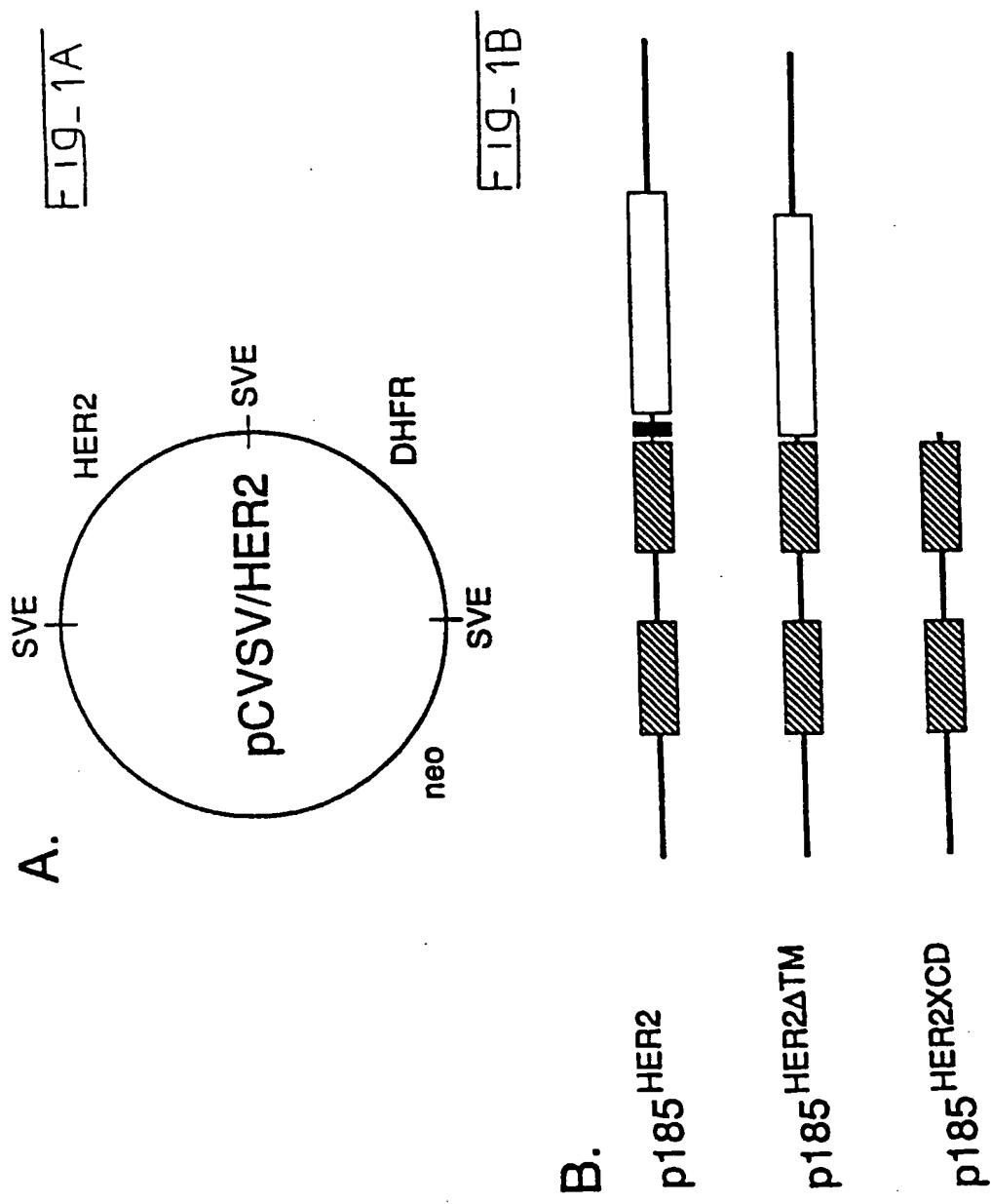
1. Verfahren zur Herstellung einer Zusammensetzung zur Verwendung bei der aktiven spezifischen Immuntherapie, umfassend die Herstellung eines extrazellulären Abschnitts des HER2-Moleküls mit zumindest 9 Aminosäuren und/oder einem Immunepitop, der im wesentlichen frei von Transmembran- und intrazellulären Abschnitten des HER2-Moleküls und in im wesentlichen reiner Form ist.
2. Verfahren nach Anspruch 1, worin der extrazelluläre Abschnitt des HER2-Moleküls eine Reinheit von zumindest etwa 99% besitzt.
3. Verfahren nach Anspruch 1 oder 2, worin der extrazelluläre Abschnitt des HER2-Moleküls den gesamten extrazellulären Abschnitt des HER2-Moleküls umfasst.
4. Verfahren nach einem der vorhergehenden Ansprüche, worin der extrazelluläre Abschnitt des

- HER2-Molécule avec un peptide, qui possède des propriétés immunogènes, conjugué.
5. Procédé selon la revendication 4, dans laquelle le peptide comprend un epitope immun.
 6. Procédé selon l'une quelconque des revendications 1 à 5, dans laquelle la composition comprend un adjuvant.
 7. Procédé selon la revendication 6, dans laquelle le peptide comprend un epitope immun.
 8. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
 9. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
 10. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
 11. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
 12. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
 13. Utilisation d'un extracellulaire de la molécule HER2, qui comprend au moins 9 acides aminés et/ou un epitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, destinée à être utilisée en immunothérapie active spécifique.
- Revendications**
- Revendications pour les Etats contractants suivants : AT, BE, CH, DE, DK, FR, GB, IT, LI, LU, NL, SE**
1. Composition comprenant une portion extracellulaire de la molécule HER2 comprenant au moins 9 aminoacides et/ou un épitope immun, pratiquement dépourvue des portions transmembranaires et intracellulaires de la molécule HER2, et sous une forme pratiquement pure, destinée à être utilisée en Immunothérapie Active Spécifique.
 2. Composition la revendication 1, dans laquelle la portion extracellulaire de la molécule HER2 a une pureté d'au moins environ 99 %.
 3. Composition suivant la revendication 1 ou la revendication 2, comprenant la portion extracellulaire totale de la molécule HER2.
 4. Composition suivant l'une quelconque des revendications précédentes, dans laquelle la portion extracellulaire de la molécule HER2 est conjuguée à un peptide ayant des propriétés immunogènes.
 5. Composition suivant la revendication 4, dans laquelle le peptide comprend un épitope immun.
 6. Composition suivant l'une quelconque des revendications 1 à 5, comprenant en outre un adjuvant.
 7. Composition suivant la revendication 6, dans laquelle l'adjuvant comprend l'un quelconque des constituants du groupe comprenant l'alun, le bacille de Calmette-Guérin (BCG), un dérivé de paroi cellulaire de BCG, Detox, Corynebacterium parvum, l'interféron gamma, l'interleukine 1, l'interleukine 2, le Syntex-1 et l'interleukine 6.
 8. Utilisation d'une portion extracellulaire de la molécule HER2 comprenant au moins 9 aminoacides et/ou un épitope immun, pratiquement dépourvu de portions transmembranaires et intracellulaires de la molécule HER2, dans la production d'une composition pour le traitement d'un patient par immunothérapie active spécifique.
 9. Utilisation suivant la revendication 8, dans laquelle la composition comprend la portion extracellulaire totale de la molécule HER2.
 10. Utilisation suivant la revendication 8 ou la revendication 9, dans laquelle la portion extracellulaire de la molécule HER2 est conjuguée à un peptide ayant des propriétés immunogènes.
 11. Utilisation suivant la revendication 10, dans laquelle le peptide comprend un épitope immun.
 12. Utilisation suivant l'une quelconque des revendications 8 à 11, dans laquelle la composition comprend un adjuvant.
 13. Utilisation suivant la revendication 12, dans laquelle l'adjuvant comprend l'un quelconque des constituants du groupe comprenant l'alun, le bacille de

Calmette-Guérin (BCG), un dérivé de paroi cellulaire de BCG, Detox, Corynebacterium parvum, l'interféron gamma, l'interleukine 1, l'interleukine 2, le Syntex-1 et l'interleukine 6.

Revendications pour l'Etat contractant suivant : ES

1. Procédé pour la production d'une composition destinée à être utilisée en immunothérapie active spécifique, comprenant la production d'une portion extracellulaire de la molécule HER2 comprenant au moins 9 aminoacides et/ou un épitope immun, pratiquement dépourvue des portions transmembranaires et intracellulaires de ladite molécule HER2, et sous une forme pratiquement pure. 5
2. Procédé suivant la revendication 1, dans lequel la portion extracellulaire de la molécule HER2 a une pureté d'au moins environ 99 %. 10
3. Procédé suivant la revendication 1 ou la revendication 2, dans lequel la portion extracellulaire de la molécule HER2 comprend la portion extracellulaire totale de la molécule HER2. 15
4. Procédé suivant l'une quelconque des revendications précédentes, dans lequel la portion extracellulaire de la molécule HER2 est conjuguée à un peptide ayant des propriétés immunogènes. 20
5. Procédé suivant la revendication 4, dans lequel le peptide comprend un épitope immun. 25
6. Procédé suivant l'une quelconque des revendications 1 à 5, dans lequel la composition comprend en outre un adjuvant. 30
7. Procédé suivant la revendication 6, dans lequel l'adjuvant comprend l'un quelconque des constituants du groupe comprenant l'alun, le bacille de Calmette-Guérin (BCG), un dérivé de paroi cellulaire de BCG, Detox, Corynebacterium parvum, l'interféron gamma, l'interleukine 1, l'interleukine 2, le Syntex-1 et l'interleukine 6. 35
8. Utilisation d'une portion extracellulaire de la molécule HER2 comprenant au moins 9 aminoacides et/ou un épitope immun, pratiquement dépourvue des portions transmembranaires et intracellulaires de ladite molécule HER2, dans la production d'une composition destinée au traitement d'un patient par immunothérapie active spécifique. 40
9. Utilisation suivant la revendication 8, dans laquelle la portion extracellulaire de la molécule HER2 comprend la portion extracellulaire totale de la molécule HER2. 45
10. Utilisation suivant la revendication 8 ou la revendication 9, dans laquelle la portion extracellulaire de la molécule HER2 est conjuguée à un peptide ayant des propriétés immunogènes. 50
11. Utilisation suivant la revendication 10, dans laquelle le peptide comprend un épitope immun. 55
12. Utilisation suivant l'une quelconque des revendications 8 à 11, dans laquelle la composition comprend un adjuvant.
13. Utilisation suivant la revendication 12, dans laquelle l'adjuvant comprend l'un quelconque des constituants du groupe comprenant l'alun, le bacille de Calmette-Guérin (BCG), un dérivé de paroi cellulaire de BCG, Detox, Corynebacterium parvum, l'interféron gamma, l'interleukine 1, l'interleukine 2, le Syntex-1 et l'interleukine 6.



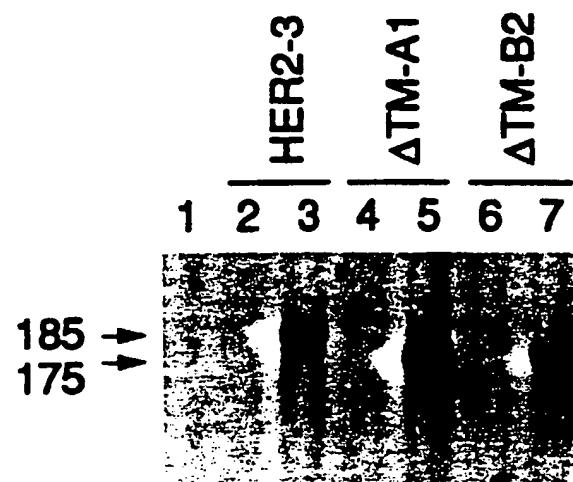


Fig - 2

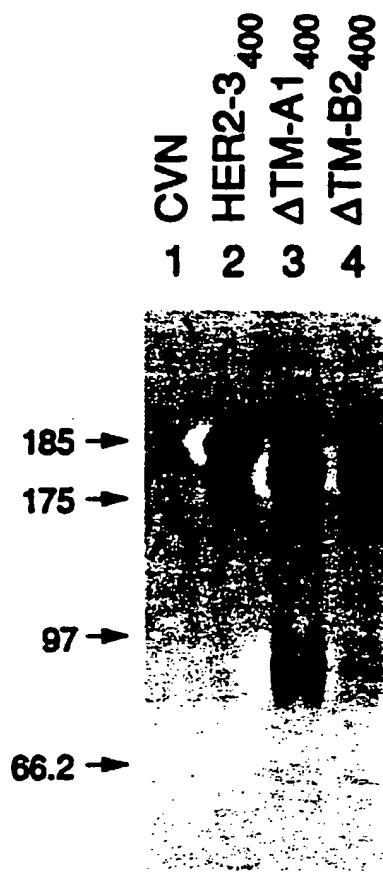


Fig - 3

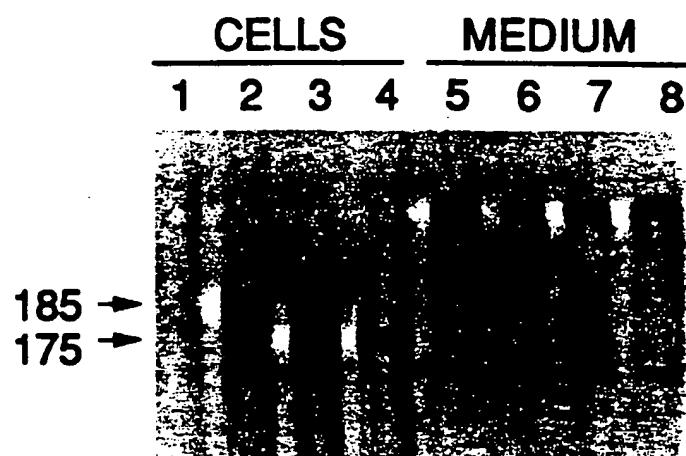


Fig- 4

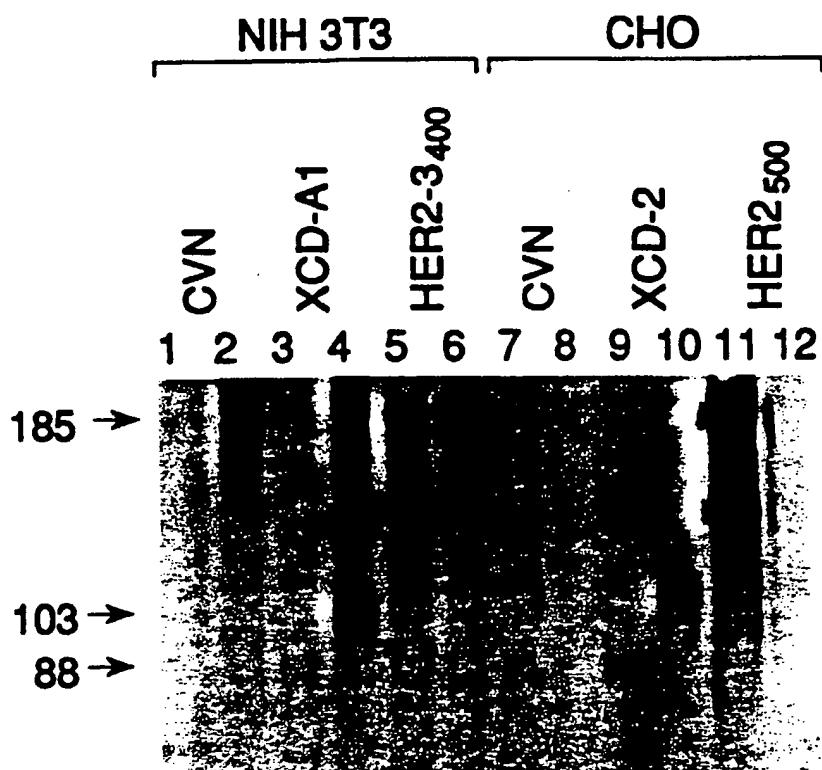
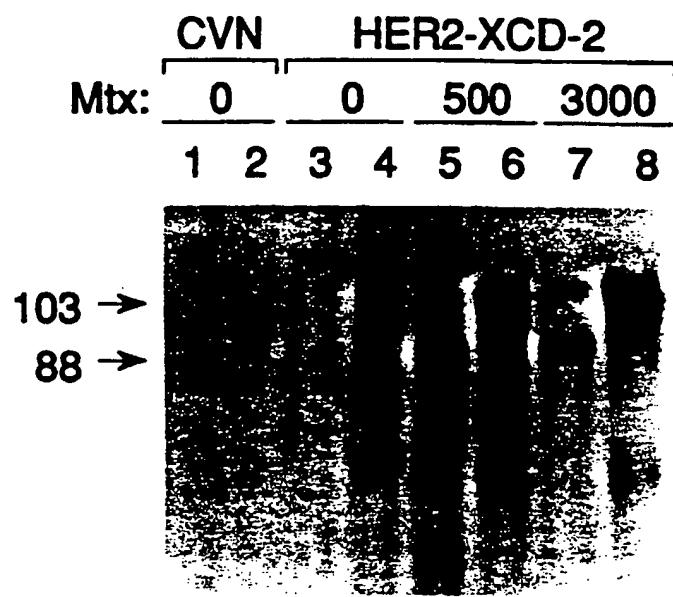


Fig - 5



F19 - 6

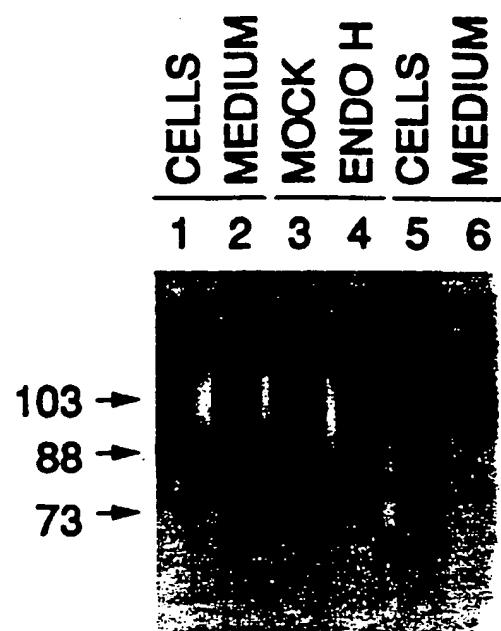


Fig - 7

Fig - 8A



Fig - 8B

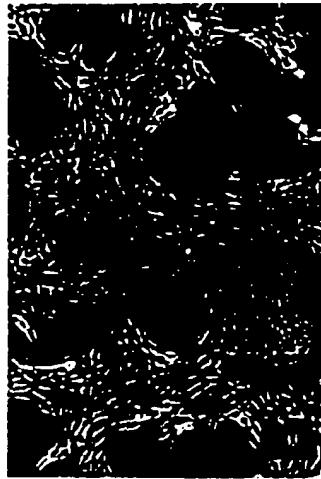


Fig - 8C



Fig - 8D

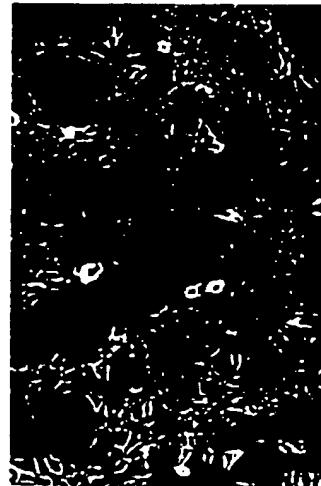


Fig - 8E



Fig - 8F

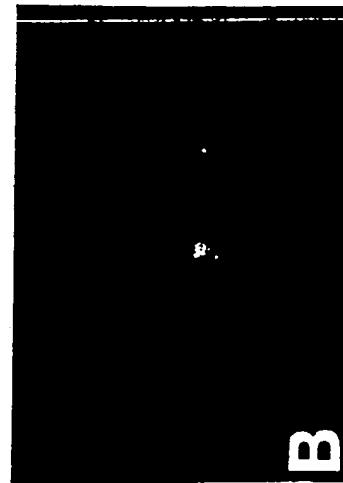


Fig - 9A



B

Fig - 9B



C

Fig - 9C



D

Fig - 9D



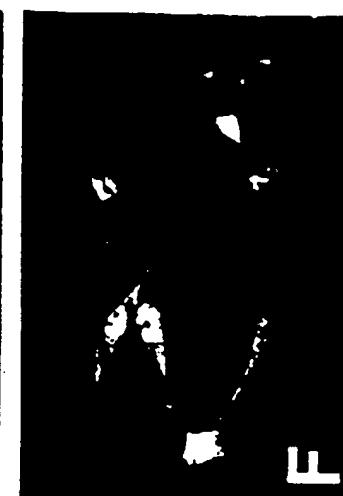
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Fig - 9E

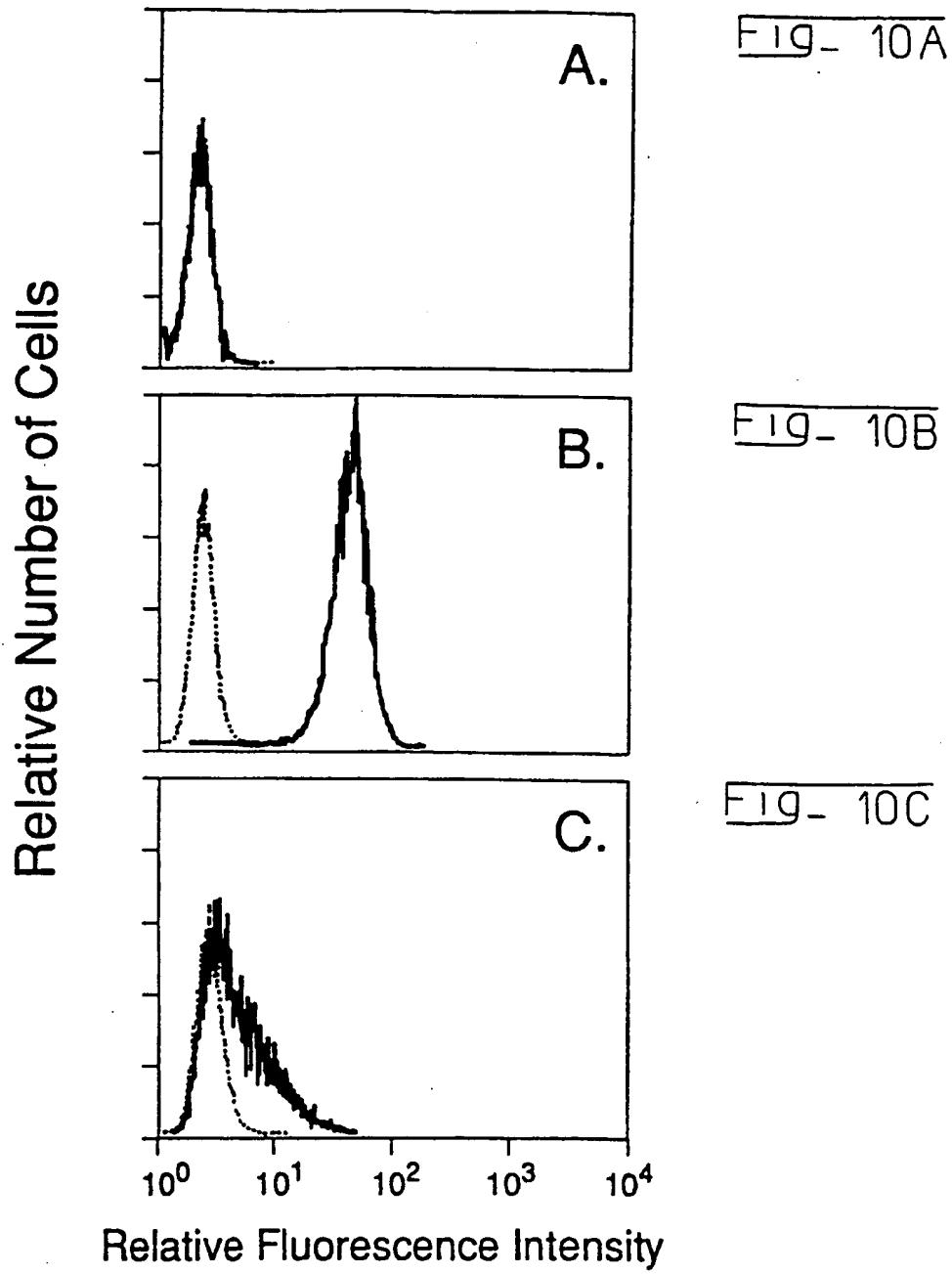


E

Fig - 9F



F



HER2-3₄₀₀ ΔTM-A1₄₀₀

	CVN	CONTROL	ENDO H	MOCK	TN	CONROL	ENDO H	MOCK	TN
1									
2									
3									
4									
5									
6									
7									
8									
9									

185 →
175 →



Fig - 11

Purification of the HER2 Extracellular Domain

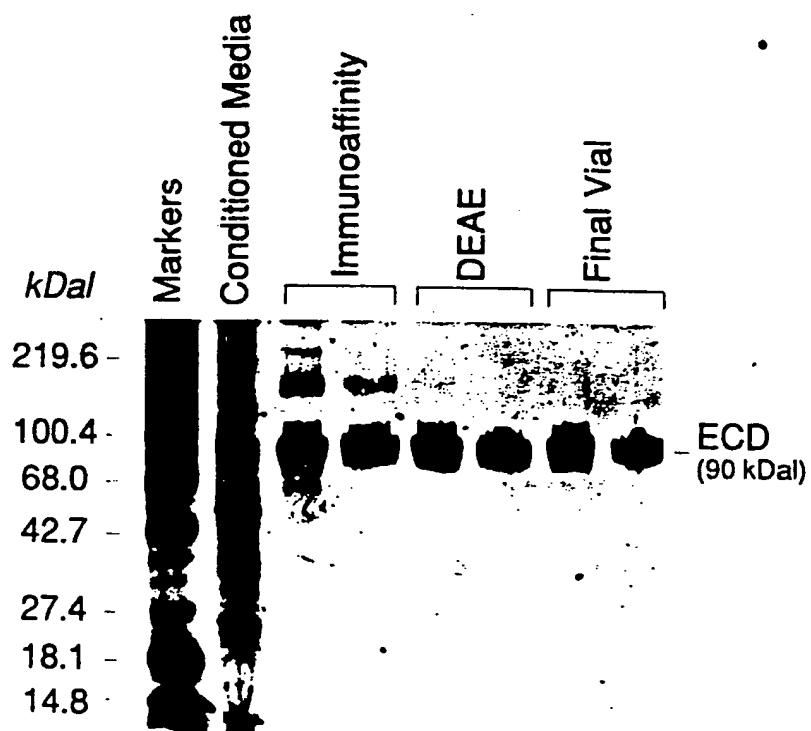


Fig - 12

FIGURE 13

1 SER THR GLN VAL CYS THR GLY THR ASP MET LYS LEU ARG LEU PRO ALA SER PRO GLU THR
 AGC ACC CAA GUG UGC ACC GGG ACA GAC AUG AAG CUG CGG CUC CCU GCC AGU CCC GAG ACC
 10
 HIS **[LEU ASP MET LEU ARG HIS LEU TYR GLN GLY CYS GLN VAL VAL GLN GLY ASN LEU GLU**
CAC CUG GAC AUG CUC CGC CAC CUC UAC GAG GGC UGC CAG GUC CAG GCA AAC CUG GAA
 20
 30
 40
 50
 60
 70
 80
 90
 100
 110
 120
 130
 140
 150
 160

LEU THR TYR LEU PRO THR ASN ALA SER LEU SER PHE LEU GLN ASP ILE GLN GLU VAL GLN
 CUC ACC UAC CUG CCC ACC AAU GCC AGC CUG UCC UUC CUG CAG GAU AUC CAG GAG GUG CAG
 GLY TYR VAL LEU ILE ALA HIS ASN GLN VAL ARG GLN VAL **[PRO LEU GLN ARG LEU ARG ILR**
GCG UAC GUG CUC AUC GCU CAC AAC CAA GUG AGG CAG GUC CCA CUC CAG AGG CUG CGG AUU
 VAL ARG GLY THR GLN LEU PHE GLU ASP ASN TYR ALA LEU ALA VAL LEU ASP ASN GLY ASP
GUG CGA CGC ACC CAG CUC UUU GAG GAC AAC UAU GCC CUG GUC CUA GAC AAU GGA GAC
 PRO LEU ASN ASN THR THR PRO VAL THR GLY ALA SER **[PRO GLY GLY LEU ARG GLU LEU GLN**
CCG CGG AAC AAU ACC ACC CCU GCU GUC ACA GCA GGG GCC UCC CCA CGA CGC CUG CGG GAG CUG CAG
 LEU ARG SER LEU THR GLU ILE LEU LYS GLY GLY VAL LEU ILE GLN ARG ASN PRO GLN LEU
CUU CGA CGC CUC ACA GAG AUC UUG AAA GGA GGG GUC UUG AUC CAG CGG AAC CCC CAG CUC
 CYS TYR GLN ASP THR ILE LEU TRP LYS ASP ILE PHE HIS LYS ASN ASN GLN LEU ALA LEU
 UGC UAC CAG GAC ACC AGC AUU UUG UGG AAG GAC AUC UUC CAC AAC AAC CAG CUG GCU CUC

FIGURE 13

THR LEU ILE ASP THR ASN ARG SER PRO CYS HIS PRO MET CYS LYS GLY
 ACA CUG AUA GAC ACC AAC CGC UCU CGG GCC UGG CAC CCC UGU UCU CCG AUG UGU AAG GGC 180
 170
 SER ARG CYS TRP GLY GLU [SER SER GLU ASP CYS GLN SER LEU THR ARG THR] VAL CYS ALA
 UCC CGC DGC UGG GGA GAG AGU UCU GAG UGU CAG AGC CUG AGC CGC ACTU GUC UGU GCC 200
 190
 GLY GLY CYS ALA ARG CYS LYS GLY PRO LEU PRO THR ASP CYS CYS HIS GLU GLN CYS ALA
 GGU GGC UGU GCC CGC UGG CGC UGG CCA CGU CCC ACU GAC UGC UGG CAU GAG CAG CAC UGU GCU 220
 210
 ALA GLY CYS THR GLY PRO LYS HIS SER ASP CYS LEU ALA CYS LEU HIS PHE ASN HIS SER
 GCC GGC UGC UGG CGC CCC AAG CAC UGU GAC UGC UGG CUG GCC UGC CUC CAC UUC AAC CAC AGU 240
 230
 GLY ILE CYS GLU LEU HIS CYS PRO ALA ILE VAL THR TYR HIS CYS LEU HIS PHE GLU SER
 GGC AUC UGU GAG CUC CAC UGG CCA CGC CGG UAU ACA UUC GUC ACC UAC AAC ACA GAC UGG UUU GAG UCC 260
 250
 MET PRO GLU GLY ARG TYR THR PHE GLY ALA SER CYS VAL THR ALA CYS PRO TYR
 AUG CCC AAU CCC GAG CGC CGG UAU ACA UUC GUC ACC UGG ACU GGU GUG UGU CCC UAC UAC 280
 270
 ASN TYR LEU SER THR ASP VAL GLY SER CYS THR LEU VAL CYS PRO ILE HIS ASN GLN GLU
 AAC UAC CUU UCU ACG GAC GUG GGA UCC ACC CUC GUC UGG CCC CUG CAC AAC CAA GAG 300
 290
 VAL THR ALA GLU [ASP GLY THR GLN ARG CYS GLU LYS CYS SER LYS] PRO CYS ALA ARG VAL
 GUG ACA GCA GAG GAU CGA ACA CAG CGG UGU GAG AAG UGG AGC AAG CCC UGU GCC CGA GUG 320
 310
 CYS TYR GLY LEU GLY MET GLU HIS LEU ARG GLU VAL ARG ALA VAL THR SER ALA ASN ILE
 UGC UAU GGU CUG GGC AUG GAG CAC UGG CGA GAG GUG AGG GCA GUY ACC AGU GCC AAA AUG AUC 340

FIGURE 13

GLN GLU PHE ALA GLY CYS LYS ILE PHE GLY SER LEU ALA PHE LEU PRO GLU SER PHE
 CAG GAG UUU GCU GGC UGG AAG AUG UUU GGG AGC CCG CUG CCA UUU CGG CCG GAC UGU 360
 ASP GLY ASP PRO ALA SER ASN THR ALA PRO LEU GLN PRO GLU GLN LEU GLN VAL PHE GLU
 GAU GGG GAC CCA GCC UCC AAC ACU GCC CCC CUC CAG CCA GAG CAG CUC CAA GUC UUU GAC 370
 THR LEU GLU GLU ILE THR GLY TYR LEU TYR ILE SER ALA TRP PRO ASP SER [LEU PRO ASP
 ACU CUG GAA GAG AUG ACA GGU UAC UCA UAC UCA GCA UGG CAC AGC CUG CCU GAC 380
 LEU SER VAL PHE GLN ASN LEU GLN VAL ILE ARG GLY ARG ILE LEU HIS ASN GLY ALA TYR
 CTC AGC GUC DUC CAG AAC CTC GAA GUA AUC CTC AAC ACC CTC AAC CTC AAC CTC AAC UAC 390
 SER LEU THR LEU GLN GLY LEU GLY ILE SER TRP LEU GLY LEU ARG SER LEU ARG GLU LEU
 UGG CUG ACC CUG CAA GGG CUG CUG AGC UGG CUG CUG CUG CTC CTC AAC CTC AAC CTC AAC CTC 400
 GLY SER GLY LEU ALA LEU ILE HIS HIS ASN THR HIS LEU CYS PHE VAL HIS THR VAL PRO
 GGC AGU GGA CTG GCC CTC AAC CAC CAU AAC ACC CAC CTC AAC CAC CTC AAC CAC CTC AAC CAC CTC 410
 TRP ASP GLN LEU PHE ARG ASN PRO HIS GLN ALA CYS HIS GLN LEU CYS ALA ARG GLY HIS CYS TRP
 UGG GAC CAG CTC UUU CGG AAC CCG CAC CAC GCU CUG CUC CAC ACU GCU CAC AAC CTC AAC CTC AAC CTC 420
 ASP GLU CYS VAL GLY GLU GLY LEU ALA CYS HIS GLN LEU CYS ALA ARG GLY HIS CYS TRP
 GAC GAG UGU GUG GGC GAG GGC CUG CCG CAC CAC CAC CAC UGG CUG CUC CAC CAC UGG CAC UGG 430
 GLY PRO GLY PRO THR GLN CYS VAL ASN CYS SER GLN GLU ARG GLY GLN GLU CYS VAL
 GGU CCA GGG CCC ACC CAG UGU GUC AAC UGC AGC CAG UUC CUU CGG GGC CAG CAC UGG CUG 440
 450
 460
 470
 480
 490
 500
 510
 520

FIGURE 13

530 540
GLU GLU CYS ARG VAL LEU GLN GLY LEU PRO ARG GLU TYR VAL ASN ALA ARG HIS CYS LEU
CAG GAA UGG CGA GUA CUG CAC GGC CUC CCC ACC GAG UAU GUG AAU GCC AGG CAC UGU UUG

 550 560
 PRO CYS HIS PRO GLU CYS GLN PRO GLN ASN GLY SER VAL THR CYS PHE GLY PRO GLU ALA
 CCG UGG CAC CCU CAG UGU CAG CCC CAG CAC UAU GGC UCA GUG ACC UGU UUU GGA CCG GAG CCU

 570 580
 ASP GLN CYS VAL ALA CYS ALA HIS TYR LYS ASP PRO PRO PHE CYS VAL ALA ARG CYS PRO
 GAC CAG UGU GUG GCC DGU GCC CAC UAU AAG GAC CCU CCC UUC UGG GUG GCC CGC UGC CCC

 590 600
 SER GLY VAL LYS PRO ASP LEU SER TYR MET PRO ILE TRP LYS PHE PRO ASP GLU GLU GLY
 AGC GGU GUG AAA CCU GAC CUC UCC UAC AUG CCC AUC UGG AAG UUU CCA GAU GAG GAG GGC

 610 620
 ALA CYS GLN PRO CYS PRO ILE ASN CYS THR HIS SER CYS VAL ASP LEU ASP ASP LYS GLY
 GCA UGG CAG CCU UGG CCC AAC AUC UGC ACC CAC DCC UGU GUG GAC CUG CAG AAC AAG GCC

 624
 CYS PRO ALA GLU
 UCC CCC GCC GAG